IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicants	:	Svend Birkelund, Gunna Christiansen, Katrine Knudsen, Per Mygind og Anne-Sofie Hebsgaard Pedersen
Serial no.	:	09/446,667
Filed	:	December 23, 1999
For	:	Surface exposed proteins from Chlamydia pneumoniae
Examiner	:	Khatol S Shahnan-Shah
Art unit	:	1645

Declaration of Svend Birkelund

1. I, Svend Birkelund, Søtoften 26, DK-8250 Egå, Denmark, in my capacity as professor at The Department of Medical Microbiology and Immunology, DK-8000 Aarhus C, Denmark, do state and declare as follows:

2. I am one of the named inventors of the above-captioned patent application. I believe that I am a person skilled in the art to which the above-captioned application pertains.

3. I have read the Office Action dated 26 September 2003. According to the Office Action subsequences of the present proteins are not enabled. The present invention relates to a protein *per se* and hence is enabled if there is any use for the claimed proteins. A subsequence of a protein can be used as a diagnostic or vaccine sub-component. To do so it is only necessary that the protein has an epitope (B-cell or T-cell epitope) and elicits an immune response. Claim 5 requires that subsequences comprise "at least one epitope of at least one of the isolated proteins".

4. As the skilled artisan in the field of immunology, I can assure that it would not take undue experimentation to identify epitopes of the proteins. It would be routine for molecular biologists to use algorithms based on local hydrophilicity, etc. to predict the location of epitopes.

5. To further emphasize that the subsequences of the proteins are enabled, we have made supplementary experiments identifying epitopes. We have determined that Omp11 (later named Pmp8) contains a CD4+ T-cell epitope by use of an ELISpot assay (Mygind et al. 2003). A protein can be fragmented different methods: 1) by use of overlapping recombinant fragments cloned into an expression vector 2) by chemical or 3) by enzymatic digests 4) synthetic peptides (Demotz et a. 1989) and the purified fragments/peptides can be used in a lymphocyte proliferation assay to map the epitopes (Demotz et a. 1989). We amplified the genes encoding Omp4-15 and expressed them in *E. coli*. The recombinant proteins were purified and tested for activation of lymphocytes. Omp11 (later Pmp8) was shown to activate lymphocytes by ELISPOT (Czerkinsky et al. 1988). The gene encoding Omp11 was then fragmented into seven overlapping fragments (Pedersen et al. 1996), the fragments were cloned into E. coli, expressed, purified and tested for activation of lymphocytes. One fragment (fragment 3) was shown to activate lymphocytes by the ELISPOT assay (Czerkinsky et al. 1988). This fragment was further analyzed. From the AA sequence 30-mer peptides with an overlap of 10 amino acid were made as described

(Petersen et al. 1992). These peptides were then used as target in the ELISPOT assay and one 30 AA long epitope was identified (377-406: AAQGQSIYFYDPIASNTTGASDVLTINQPD). When immunizing mice with this peptide prior to a *C. pneumoniae* lung challenge mice were partially protected compared to unimmunized mice and spleen cells from the infected mice were shown to be activated by this peptide in the IFN-g ELISPOT assay.

6. Thus in addition to the general understanding that it would be routine for molecular biologists to use algorithms based on local hydrophilicity, etc. to predict the location of epitopes, we have now presented supplementary experiments which show that it neither would require undue experimentation to identify epitopes and detect the concomitant protective immune response, and hence the subsequences of the present invention are enabled.

7. Mygind, T., S. Birkelund, E. Falk and G. Christiansen. 2001. Evaluation and real-time quantitative PCR for identification and quantification of Chlamudia pneumoniae by comparison with immunohistochemistry. J Microbiol Methods. 46, 241-51.

Caldwell HD, Kromhout J, Schachter J. Related Articles, Links Abstract Purification and partial characterization of the major outer membrane protein of Chlamydia trachomatis. Infect Immun. 1981 Mar; 31(3): 1161-76.

Mygind T, BB Vandahl, AS Pedersen, P Hollsberg, S Birkelund and G Christiansen. 2003. Identification of an in vivo CD4+ T-cell-mediated response to polymorphic membrane proteins of Chlamydia pneumoniae. FEMSIM 1640: 1-9.

Pedersen LB, Birkelund S, Holm A, Østergaard S, Christiansen G. The 18 Kilodalton Chlamydia trachomatis Histone H1-Like Protein (Hc1) Contains a Potential N-terminal Dimerization Site and a C-terminal Nucleic Acid-Binding Site. J Bacteriol 1996, 178, 994-1002.

Petersen JW, Holm A, Ibsen PH, Haslov K, Capiau C, Heron I. Identification of human T-cell epitopes on the S4 subunit of pertussis toxin. Infect Immun. 1992 Oct;60(10):3962-70.

Demotz S, Lanzavecchia A, Eisel U, Niemann H, Widmann C, Corradin G. 1989. Delineation of several DR-restricted tetanus toxin T cell epitopes. J Immuno l142:394-402.

8. I further declare that all statements made herein of my own knowledge are true and further that the statements were made with the knowledge that wilful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such wilful false statements may jeopardise the validity of the application or any patent issued thereon.

Dated: 25/8-2004 Signature: Svend Birkelund

Svend Birkelund

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicants	:	Svend Birkelund, Gunna Christiansen, Katrine Knudsen, Per Mygind og Anne-Sofie Hebsgaard Pedersen
Serial no.	:	09/446,667
Filed	:	December 23, 1999
For	:	Surface exposed proteins from Chlamydia pneumoniae
Examiner	:	Khatol S Shahnan-Shah
Art unit	:	1645

Declaration of Svend Birkelund

1. I, Svend Birkelund, Søtoften 26, DK-8250 Egå, Denmark, in my capacity as professor at The Department of Medical Microbiology and Immunology, DK-8000 Aarhus C, Denmark, do state and declare as follows:

2. I am one of the named inventors of the above-captioned patent application. I believe that I am a person skilled in the art to which the above-captioned application pertains.

3. I have read the paper by Melgosa *et al.* FEMS Microbiology Letters 112 (1993) 199-204. In this paper Melgosa *et al.* describes for the first time that Chlamydia pneumoniae contains a protein of 98-kDa present in the Chlamydia outer membrane complex (COMC).

4. It is important to note that Melgosa *et al.* did not isolate the 98-kDa protein from the outer membrane complex of Chlamydia pneumoniae, but merely showed the presence of the protein using separation by electrophoresis. Thus, Melgosa *et al.* do not teach isolated proteins.

5. In fact Melgosa *et al.* had no idea that the 98-kDa protein which they showed the presence of represented a mixture of proteins. Because the 98-kDa protein was a mixture of the proteins, it was not possible to obtain an N-terminal sequence the 98-kDa protein from the gel since there would have been contamination from the other proteins represented by the 98-kDa protein. Furthermore, it would have been impossible to identify and clone the gene from knowledge obtained from an N-terminal amino acid sequence because no genome sequence was available. Consequently, Melgosa *et al.* did not isolate any of the chlamydial proteins of the present invention. In my opinion we had to employ inventive skills to achieve the important result that the 98-kDa protein disclosed by Melgosa *et al.* in fact was a mixture of several proteins.

6. Melgosa *et al.* obtained the results in two ways:

1) Radioactive labeling of all chlamydial proteins during cultivation, harvesting of the chlamydiae and separation of COMC according to Caldwell et al. 1981. Proteins present in the purified COMC were solubilized in SDS sample buffer, separated by SDS-PAGE and the proteins were visualized on X-ray films. In addition to bands presenting known proteins (MOMP, Omp2 and Omp3) a 98-kDa protein band was seen.

2) COMC proteins were separated by SDS-PAGE as described in 1) the proteins were transferred to nitrocellulose membranes and reacted with antibodies generated by immunization of rabbits with purified C. pneumoniae. The immunoblotting showed that MOMP, Omp2, Omp3 and the 98 kDa protein were recognized by the antibodies.

7. Melgosa *et al.* thus showed the presence of a 98-kDa protein in C. pneumoniae COMC but did not characterize the protein further, did not isolate the protein and did not identify the gene encoding the protein.

8. In contrast, the present application presents isolated peptides according to the amino acid sequence of each of the cloned and sequenced gens encoding 98 kDa proteins. Such peptides were obtained by cloning each gene separatelyinto an expression vector system, expressing the peptides in Escherichia coli and purifying the peptides. Thus, each peptide was obtained separately being free of any other chlamydial protein.

To be able to isolate the peptides we employed a rather different and inventive approach.

9. In order to obtain the gene(s) encoding the 98-kDa protein we first immunized a rabbit with COMC solubilized in SDS buffer. We then made a genomic library with C. pneumoniae DNA fragmented by cleavage with the restriction endonuclease SauIIIA. The fragments were cloned into the vector system pEX1,2,3, transferred into Escherichia coli, and after rescue the E. coli were plated on agar plates. The E. coli colonies were transferred onto nitrocellulose membranes and reacted with our anti-COMC antibody described above. Colonies containing a plasmid encoding fragments of the proteins that antibodies were generated against were blue, and these colonies were streaked so that single clones were obtained. From each of these clones plasmid DNA was purified, and the insert fragments were sequenced. The sequences were compared to gene bank data and clones containing fragmented MOMP, Omp2 and Omp3 were not selected for further use.

10. The clones containing unknown sequences were used to probe another genomic library of C. pneumoniae DNA that was constructed to contain large fragments of DNA. From this library large fragments were selected and bidirectionally sequenced. From these sequences genes encoding 98-kDa proteins were obtained. The genes were then amplified by PCR, cloned into an expression system and proteins were purified. The recombinant 98-kDa proteins were used to immunize rabbits and the resulting antibodies recognized the 98-kDa COMC band in immunoblotting.

11. We thus showed that the 98-kDa protein band contained many 98-kDa proteins all of which belonged to a protein family with specific features (GGAI repeats), and that these proteins are localized in COMC in the 98-kDa protein band. We thus identified and characterized a new Omp gene family (the Pmp family). We purified the proteins individually as recombinant proteins and showed the proteins to be present in COMC. We employed inventive skills to achieve the important result that the 98-kDa protein disclosed by Melgosa *et al.* in fact was a mixture of several proteins.

The isolation of the peptides is very important for vaccine use, since the peptide for such use must be purified sufficiently to be pharmaceutically acceptable. Accordingly, the protein according to the invention must be free of any other chlamydial protein.

12. I further declare that all statements made herein of my own knowledge are true and further that the statements were made with the knowledge that wilful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001

of Title 18 of the United States Code and that such wilful false statements may jeopardise the validity of the application or any patent issued thereon.

Dated: 25/3-2004 Signature: Svend Birkelund

Svend Birkelund

ELSEVIER

FEMS Microbiology Letters 203 (2001) 153–159

FEMS MICROBIOLOGY Letters

www.fems-microbiology.org

Differential expression of Pmp10 in cell culture infected with *Chlamydia pneumoniae* CWL029

Anna Sofie Pedersen [a,*], Gunna Christiansen [b], Svend Birkelund [a,b]

[a] *Loke Diagnostics ApS, Science Park Aarhus, Gustav Wieds Vej 10 C, DK-8000 Aarhus C, Denmark*

[b] *Department of Medical Microbiology and Immunology, The Bartholin Building, University of Aarhus, DK-8000 Aarhus C, Denmark*

Received 11 May 2001; accepted 8 July 2001

First published online 29 August 2001

Abstract

The complete genome of *Chlamydia pneumoniae* contains a total of 21 genes encoding polymorphic membrane proteins (Pmp). From this large Pmp family three genes, *pmp8*, *pmp10* and *pmp11*, were cloned and antibodies against recombinant full-length Pmp proteins were produced. Indirect immunofluorescence microscopy of HEp-2 cells infected with *C. pneumoniae* CWL029 was performed with the Pmp antibodies in combination with a *Chlamydia*-specific anti-lipopolysaccharide (LPS) antibody. This double staining technique clearly showed that expression of Pmp10 was differential. Additional double staining with monoclonal antibodies to the surface of *C. pneumoniae* elementary bodies and the anti-LPS antibody resulted in identification of seven monoclonal antibodies that reacted identically to the Pmp10 antibody indicating that Pmp10 is an immunodominant protein. Finally, the molecular mechanism responsible for differential expression is suggested to be variation in the guanine residues in the polyG tract of *pmp10*.

Keywords: *Chlamydia pneumoniae*; Polymorphic membrane protein; Double staining technique; Indirect immunofluorescence microscopy; Monoclonal antibody

1. Introduction

Chlamydia pneumoniae is an obligate intracellular bacterium and a common human pathogen causing infection of the upper and lower respiratory tract [1]. The *Chlamydia* species are characterized by a biphasic developmental cycle and the genus-specific, surface-localized lipopolysaccharide (LPS) epitope. When treating *Chlamydia* elementary bodies (EB) with sarcosyl, an insoluble fraction is obtained. This fraction is defined as COMC, the *Chlamydia* outer membrane complex. Besides LPS, the well characterized components of COMC are the major outer membrane protein (MOMP), outer membrane protein 2 (Omp2) and outer membrane protein 3 (Omp3). Expression of additional outer membrane proteins, referred to as polymorphic membrane proteins (Pmp), has been described in *C. pneumoniae* [2–4] and *C. psittaci* [5,6]. In *C. trachomatis* PmpE, PmpG and PmpH have been identified by mass spectrometry [7,8]. In both *C. trachomatis* and *C. psittaci* the Pmps are made late in the developmental cycle.

The recent genome sequences [9–12] have revealed 21 *pmp* genes in *C. pneumoniae* and nine in *C. trachomatis*. Common for the Pmps are the tetrapeptide GGA(I/V/L) motif repeated several times in the N-terminal part. The C-terminal half is characterized by conserved tryptophans and a carboxy-terminal phenylalanine. A signal peptide leader sequence is predicted in 20 *C. pneumoniae* Pmps [13], which indicates an outer membrane localization. Pmp10 and Pmp11 contain a signal peptidase II cleavage site [2] suggesting lipid modification. The *C. pneumoniae* Pmps comprise high molecular mass proteins of about 100 kDa (average length of 930 aa). Pmp6, Pmp20 and Pmp21 have primary sequence sizes of 1408, 1724 and 1610 amino acids. Pmp12 is a truncated version of only 514 aa as it lacks the C-terminal part shared among other full-length Pmps, and in four genes, *pmp3*, *pmp4*, *pmp5* and *pmp17*, a frameshift mutation results in prematurely terminated gene products [14]. A full-length *pmp10* gene product is produced in strains CWL029, AR39 and TW183 [2,15] whereas the CWL029 *pmp10* sequence determined by Kalman et al. [10] is out of reading frame.

* Corresponding author. Tel.: +45 8620 5141; Fax: +45 8620 1222.
E-mail address: annas@biobase.dk (A.S. Pedersen).

0378-1097/01/$20.00
PII: S0378-1097(01)00341-X

The *C. pneumoniae pmp* genes represent 17.5% of the chlamydia-specific coding capacity [16] and they are all transcribed during chlamydial growth [4] but the function of Pmps remains unknown. The in vivo expression of Pmps in infected HEp-2 cell culture has been investigated by Christiansen et al. [14] who showed that antibodies against recombinant Pmp1, 2, 4 and Pmp8–11 reacted well in indirect immunofluorescence microscopy (IMF) whereas antibodies to Pmp5 and Pmp12 failed to react. By 2-D PAGE and mass spectrometry Vandahl et al. [3] have identified 10 *C. pneumoniae* full-length Pmps (Pmp2, 6, 7, 8, 10, 11, 13, 14, 20 and 21) that were expressed in EB during cultivation, but no evidence of expression of the truncated *pmp5* and *pmp12* was found.

Efforts to identify proteins comprising the surface of *C. pneumoniae* EB have been impeded due to a dominance of conformational epitopes [17]. Consequently, attempts to identify surface-exposed antigenic determinants by immunoblotting with *C. pneumoniae* monoclonal antibodies (mAb) have been unsuccessful [18,19]. In contrast to other *Chlamydia* species, no linear epitopes were found in *C. pneumoniae* MOMP [17,20] but a conformational surface-exposed epitope was recently detected [21]. In 1999 Knudsen et al. [2] showed that *C. pneumoniae* Pmp11 contained epitopes exposed on the surface. Immunoblotting with recombinant Pmp11 verified that mAb Cp24.1.44, a *C. pneumoniae*-specific monoclonal antibody against the EB-surface [18], was directed to epitopes of Pmp11. Immunoelectron microscopy performed with mAb Cp24.1.44 demonstrated surface binding of the antibody to the reticulate body, EB and COMC. Furthermore, previous results have indicated differential expression of *pmp10* [22,23]. As these reports provide the possibility that expression of homologous Pmps is switched from on to off, the aim of this study was to analyze *C. pneumoniae*-infected cell culture for differential expression of Pmp8, Pmp10 and Pmp11. Inclusions were double-stained as monoclonal and polyclonal antibodies in combination were used for IMF analysis. By the double staining technique 11 monoclonal antibodies produced against *C. pneumoniae* EB [18] were applied for additional characterization of Pmp10. Finally, a molecular mechanism leading to differential expression of Pmp during chlamydial growth in cell culture was proposed.

Table 1
Primers used for PCR amplification of *C. pneumoniae pmp8, pmp10* and *pmp11*

Target gene	Primer sequence 5′ to 3′
pmp8 (fw)	**GACGACGACAAGAT**GATTGCAACTTACGGAG
pmp8 (rv)	**GAGGAGAAGCCCGGT**CTAGAATGAGTATCTTAGCCCAC
pmp10 (fw)	**GACGACGACAAGAT**GTGTTCCACTGTTTTTGCTGC
pmp10 (rv)	**GAGGAGAAGCCCGGT**CTAGAATTGGAACTTACCCCC
pmp11 (fw)	**GACGACGACAAGAT**GAAGACTTCGATTCCTTGGGTTTT AGTTTCC
pmp11 (rv)	**GAGGAGAAGCCCGGT**TAGAATCGGAGTTTGGTACCAAC ATCTACATTG

Nucleotides in bold are the pET-30 LIC-specified sequence.
fw: forward primer; rv: reverse primer.

2. Materials and methods

2.1. C. pneumoniae strain and cultivation

C. pneumoniae (CDC/CWL029/VR1310), purchased from the American Type Culture Collection (Rockville, MD, USA), was grown in HEp-2 cells (American Type Culture Collection) as described [24].

2.2. Cloning and expression of recombinant Pmps (rPmp)

Primers used for cloning of Pmp8, Pmp10 and Pmp11 were from DNA Technology (Aarhus, Denmark). All primers were designed with LIC-specific sequences as illustrated in Table 1. Ligation-independent cloning into the pET-30 LIC vector (Novagen, Madison, WI, USA) was done as described by the manufacturer. Control sequencing verified a correct *pmp* insert. Expression of a His-tagged fusion protein was induced with 1 mM IPTG for 2 h at 37°C. Fusion proteins were purified by affinity chromatography on HiTrap nickel columns (Amersham Pharmacia).

2.3. Production of rPmp antibodies

Recombinant Pmps were dialyzed against three volumes of 1 l phosphate-buffered saline (PBS, pH 7.4) containing 1 mM EDTA. 10–50 μg were used for i.m. immunization (antigen dissolved in PBS and emulsified in Freund's incomplete adjuvant) of New Zealand White rabbits on days 1, 8 and 15 and i.v. (antigen dissolved in PBS) on days 29, 36 and 43. Rabbits were bled on day 60.

2.4. Indirect immunofluorescence microscopy

Antibodies used for IMF are listed in Table 2. A monolayer of HEp-2 cells grown on glass coverslips was infected with 0.5 or with twofold dilution series up to 0.016 *C. pneumoniae* inclusion-forming units per cell. Cells were

Table 2
Antibodies used for IMF

Antibody	Antigen (cloned amino acids)
pAb 201	Pmp8 (21–931)
pAb 203	Pmp10 (20–929)
pAb 195	Pmp11 (1–929)[a]
pAb 198	*C. pneumoniae* OMC[a]
mAb 15.1	*Chlamydia* LPS[b]

[a]Reference [2].
[b]Reference [25].

methanol-fixed 72 h after infection and incubated with a mixture of polyclonal (pAb) and monoclonal (mAb) antibodies diluted 1:200 and 1:5, respectively. Fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit (Jackson ImmunoResearch Laboratories) and rhodamine red-conjugated goat anti-mouse (Jackson ImmunoResearch Laboratories) secondary antibodies were used in a 1:100 dilution. The IMF procedure was done as described previously [24]. Examination of inclusions was performed on a Leitz DMRBE fluorescence microscope (Leica Mikroskopie und Systeme GmbH, Wetzlar, Germany). Objectives of 100× and 40× magnification and filters for visualization of either red, green or both colors were applied.

2.5. pEX expression clones

The pEX1-1, pEX1-2 and pEX1-6 clones were generated as described by Knudsen et al. [2]. Expression of fusion proteins, done as previously described [26], was analyzed by SDS–PAGE [2].

2.6. High fidelity PCR and sequencing

Primers used for the PCR reaction and sequencing were from DNA Technology (Aarhus, Denmark). The polyG region of *pmp10* was amplified from *C. pneumoniae* CWL029 genomic DNA as primers were designed to anneal 130–140 bp from the string of guanines. The primers 5′-GGGAATAAATCGAGCGC-3′ and 5′-GGTGTAGTTGCAACAATGGC-3′ resulted in a product of 491 bp (covers bp 568–1058 of *pmp10*). The Expand® High Fidelity PCR System (Roche, Mannheim, Germany) was used for PCR reactions. The High Fidelity enzyme mix contains the thermostable Taq DNA polymerase and Pow DNA polymerase. PCR was done in accordance with the manufacturer's instructions. DNA sequencing was performed with an ABI Prism 377 DNA Sequencer (Perkin Elmer) and templates for sequence reactions were: (i) plasmid DNA from the pEX1-1, pEX1-2 and pEX1-6 clones from the original *C. pneumoniae* CWL029 expression library [2] and (ii) the 491-bp PCR product of *C. pneumoniae pmp10*. Sequence reactions were performed with the Terminator Ready Reaction Mix (Perkin Elmer, Applied Biosystems, UK) mixed with 0.5 μg template DNA, 1 pmol of each primer, and thermocycled. Primers for sequencing of the PCR product were 5′-CGCAGGAAATGGAGGAGC-3′ and 5′-GGTGTAGTTGCAACAATGGC-3′. Sequencing of plasmids was performed with primers specific for the pEX vector.

2.7. Accession number

The accession number is AJ133034 (nucleotide and amino acid sequences of *C. pneumoniae* CWL029 *omp4* (*pmp11*), *omp5* (*pmp10*) and *omp11* (*pmp8*)).

3. Results and discussion

3.1. Differential expression of Pmp10

To determine whether Pmp8, Pmp10 or Pmp11 were differentially expressed, IMF was performed on *C. pneumoniae*-infected HEp-2 cells fixed with methanol 72 h post infection. After fixation, infected host cells were subjected to the double staining technique where cells were incubated with polyclonal Pmp antibodies against Pmp8 (anti-Pmp8, pAb 201), Pmp10 (anti-Pmp10, pAb 203) and Pmp11 (anti-Pmp11, pAb 195) each applied in combination with mAb 15.1 (anti-LPS) [25] that was used for detection of all inclusions containing *Chlamydia*. Primary polyclonal antibodies were visualized with a FITC-conjugated antibody (green fluorescence) and monoclonal antibodies were visualized with rhodamine red-conjugated (red fluorescence) antibodies. To distinguish between antibody reactions three filters for visualization of either green, red or both colors were used for microscopy. Inclusions containing bacteria that were immunostained with both antibodies appeared yellow when a filter allowing both colors to be visible was used.

IMF analysis with anti-LPS mixed with either Pmp8 or Pmp11 antibodies demonstrated an identical staining pattern as the pattern of red-stained inclusions overlapped the pattern of green colored inclusions. Fig. 1A,B and C,D show no variation in expression of Pmp8 and Pmp11 as the number and location of red and green inclusions were identical. This strongly indicates that Pmp8 and Pmp11 are expressed in all *C. pneumoniae* EB, which is in agreement with results obtained by Vandahl et al. recently [3].

A different reaction pattern resulted from double staining performed with anti-Pmp10 and anti-LPS antibodies. Two examples are shown in Fig. 1E,F where the filter for visualization of both colors was used. Compared to anti-LPS, the anti-Pmp10 reaction demonstrated that only part of inclusions or a small fraction of bacteria within a single inclusion (Fig. 1F, arrow) reacted with anti-Pmp10. Therefore, some inclusions appeared red since the majority of EB in those inclusions did not express Pmp10. Thus, these IMF results show that Pmp10 was differentially expressed in EB relative to the LPS. In order to analyze the Pmp10 switching, a twofold dilution series of the infection dose was made. At the highest dilution (1 inclusion/10 cells) differential expression of Pmp10 was still seen in many of the inclusions (Fig. 1G, arrow), illustrating that only few bacteria expressed Pmp10. This result therefore indicates that within a single inclusion originating from infection with one EB, differential expression of Pmp10 had been generated during development of the inclusion. Furthermore, the differential staining pattern was not a result of different stages of the developmental cycle because the differential appearance was seen in inclusions of the same size.

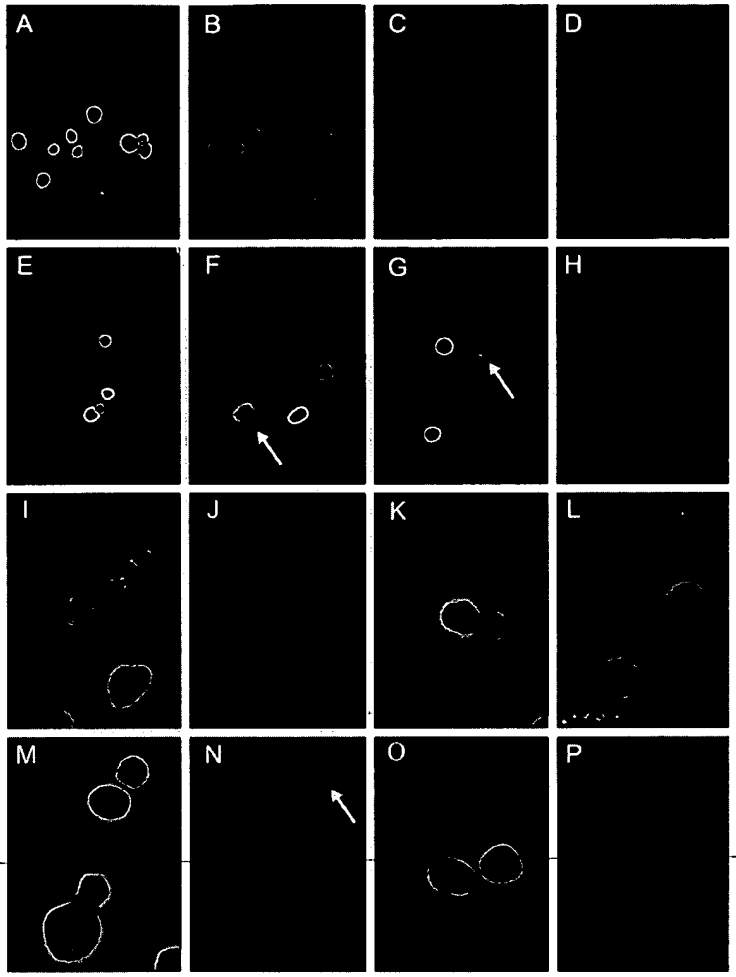

Fig. 1. IMF of methanol-fixed, double-stained HEp-2 cells infected with *C. pneumoniae* CWL029. Antibodies used for double staining were: A and B, anti-Pmp8 and anti-LPS; C and D, anti-Pmp11 and anti-LPS; E–H, anti-Pmp10 and anti-LPS; I and J, anti-Pmp10 and mAb Cp26.4.23; K and L, anti-Pmp10 and mAb Cp18.4; M and N, anti-COMC and mAb Cp26.4.23; O and P, anti-COMC and mAb Cp18.4. The secondary antibodies were rhodamine red-conjugated goat anti-mouse and FITC-conjugated goat anti-rabbit antibodies. For A, B, E and F a 32× magnification was used. The magnification in the remaining panels was 80×.

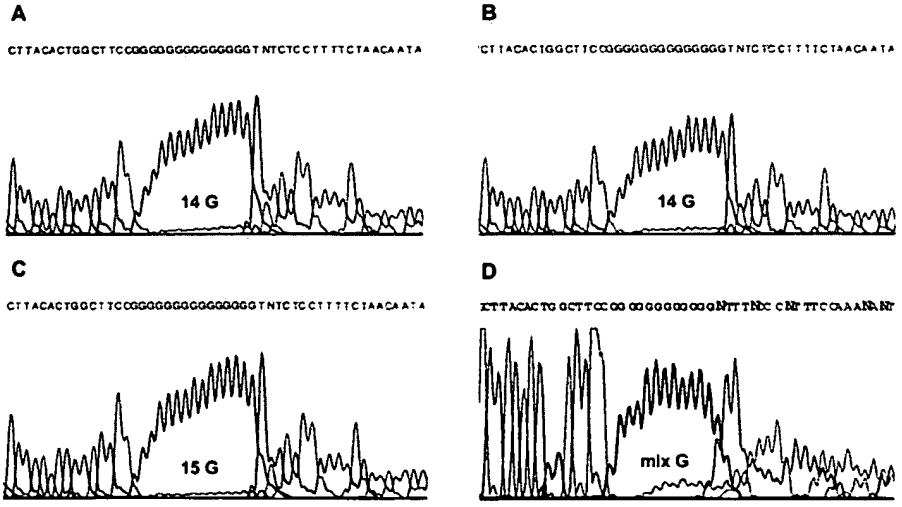

Fig. 2. Results of DNA sequencing. The plasmid *pmp10* sequences showed that the polyG tract of (A) pEX1-1, (B) pEX1-2 and (C) pEX1-6 contained strings of 14, 14 and 15 guanines respectively. D: The genomic *C. pneumoniae* CWL029 DNA sequence revealed an out of frame sequence after the poly-G tract.

The presented data are the first to confirm the on/off switching in the expression of Pmps noted previously [22,23]. An alternating expression of homologous Pmps will lead to changes of the protein composition at the chlamydial outer membrane and contribute to generation of surface variability. In this study the Pmp expression was examined late (72 h after infection) in the *Chlamydia* developmental cycle which corresponds to very recent reports concluding that *C. psittaci* and *C. trachomatis* Pmps were being synthesized in the late developmental phase [6,8]. Finally, it must be mentioned that the *pmp10* gene is located in a cluster together with *pmp8*, *pmp11* and seven other *pmps*. Within this cluster all the genes have the same direction of transcription except for *pmp10*, which is placed tail to tail with *pmp11* being transcribed in the opposite direction. Consequently the *pmp10* transcript is monocistronic, and the probability of another *pmp* gene product being produced together with *pmp10* is negligible.

3.2. Characterization of Pmp10 by monoclonal antibodies

In a recent study, IMF performed on formaldehyde-fixed cell culture infected with *C. pneumoniae* has shown that Pmp10 (Omp5) and Pmp11 (Omp4) contained surface-exposed epitopes [2]. Therefore, a panel of 11 monoclonal antibodies, generated against *C. pneumoniae* EB, which reacted with surface-exposed epitopes different from LPS [18], were used to compare reactivity with Pmp10 by the double staining technique in IMF. Each monoclonal antibody was mixed with the polyclonal anti-Pmp10 antibody, and by IMF eight monoclonal antibodies had staining pattern identical to that of anti-Pmp10 as summarized in Table 3. These results are exemplified by the anti-Pmp10 and mAb Cp26.4.23 staining (Fig. 1I,J). Two examples of a different reaction pattern are the combination of mAb Cp18.4 and anti-Pmp10 shown in Fig. 1K,L. The green (and not yellow) appearance (Fig. 1K) was due to an abundant expression of Pmp10.

The monoclonal antibody reacting identically to anti-Pmp10 should not react with all inclusions. In order to detect all *C. pneumoniae* inclusions when IMF was done with the monoclonal antibodies, a polyclonal antibody against SDS-denatured *C. pneumoniae* OMC, pAb 198 [2] (anti-COMC), was used. All inclusions were stained with anti-COMC (Fig. 1M) whereas with mAb Cp26.4.23 one inclusion was seen not to be stained (arrow in Fig. 1N). As control mAb Cp18.4 stained all inclusions (Fig. 1O,P). Table 3 lists the IMF results of the anti-COMC reactions when combined with monoclonal antibodies.

In summary, IMF performed with the double staining technique has identified seven *C. pneumoniae* monoclonal antibodies with a staining pattern identical to that of anti-Pmp10. These were Cp5.4, Cp13.4.30, Cp26.4.23, Cp28.27, Cp30.1.42, Cp36.2 and Cp38.3.33 (Table 3). The epitopes recognized by mAbs Cp18.4 and Cp20.2.26 were present at a surface protein that was not differentially expressed, while mAb Cp24.1.44 has previously been characterized as a Pmp11-specific antibody [2]. The double staining with mAb Cp24.1.44 mixed with anti-Pmp10 clearly demonstrated that Pmp10 surface-exposed epitopes were not identical to those recognized by mAb Cp24.1.44. The staining pattern was, however, also different from that of anti-COMC. Thus, here we have succeeded in identifying seven monoclonal antibodies with a reactivity identical to that of anti-Pmp10, and we therefore conclude that Pmp10 is an immunodominant protein in *C. pneumoniae*. With respect to data obtained by Vandahl et al. [3], Pmp10 comprises as much as 1.1% of the total EB protein amount, which is the highest value for the Pmps identified in that study. Given the differential expression of Pmp10, the significant abundance in EB and the existence of surface-exposed epitopes, we suggest that *C. pneumoniae* Pmp10 is an important antigenic determinant likely to be involved in protection against the host immune system. In the OMP90 gene family of *C. psittaci* the encoded proteins were found to be major immunogens in post-abortion sheep sera [27] and the amino-terminal part of OMP90A was exposed at the surface of EB [28].

Table 3
Summary of the staining patterns obtained from IMF performed with the double staining technique

C. pneumoniae mAb[a]	IMF performed with anti-Pmp10 and mAbs	IMF performed with anti-COMC and mAbs
Cp5.4	**identical**	**different**
Cp13.4.30	**identical**	**different**
Cp18.4	different	identical
Cp20.2.26	different	identical
Cp24.1.44	different	different
Cp26.4.23	**identical**	**different**
Cp28.27	**identical**	**different**
Cp30.1.42	**identical**	**different**
Cp31.2	identical	not determined
Cp36.2	**identical**	**different**
Cp38.3.33	**identical**	**different**

[a]Reference [18].

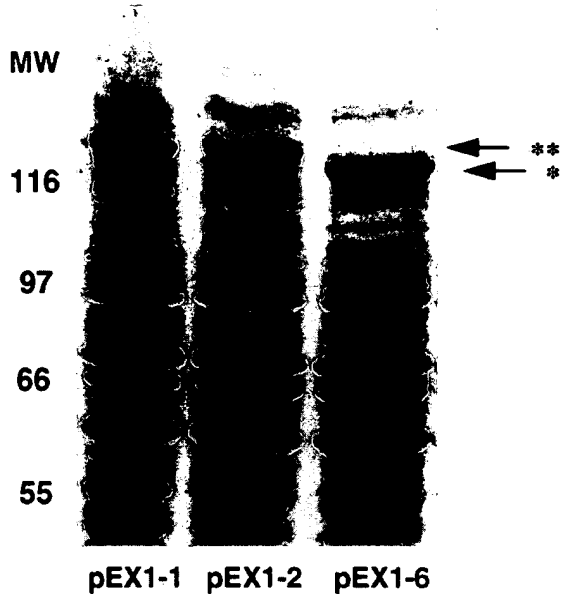

Fig. 3. SDS-PAGE separation and Coomassie brilliant blue staining of induced *E. coli* cell lysate containing cro-β-galactosidase-Pmp10 fusion proteins. The size of cro-β-galactosidase is 117 kDa. Calculated sizes of pEX1-1 and pEX1-2 were 17.4 kDa, whereas pEX1-6 had a size of 12.0 kDa. Arrows: ** denotes full-length protein; * denotes truncated protein. Standard molecular masses in kDa are shown.

3.3. The polyG tract of pmp10

The genome sequences of the CWL029, AR39 and J138 strains [10–12] show little genetic variation of the *pmp* genes. However, genome sequencing has demonstrated both intrastrain and interstrain variation in the number of guanine (G) residues in the *pmp10* polyG tract. Based upon the sequence of CWL029 *pmp10* published by Knudsen et al. [2], a string of 14 guanine residues was identified. This was identical to the number found in the AR39 strain [11] whereas the sequence determined by Kalman et al. [10] was out of reading frame due to a *pmp10* polyG tract of 13 residues. Therefore, further sequencing of the region spanning the *pmp10* polyG tract was performed in order to analyze for variation in the number of guanine residues. Sequencing was carried out on a 491-bp PCR product amplified from genomic *C. pneumoniae* CWL029 DNA and three different pEX plasmids from the expression library [2] which all encoded an identical part of *pmp10* (amino acids 185–365). The results of sequencing (Fig. 2A–C) show that the polyG tracts of pEX1-1, pEX1-2 and pEX1-6 contain 14, 14 and 15 guanines, respectively. Sequencing of the corresponding *pmp10* region, amplified from genomic DNA, demonstrated a sequence with a variable number of G residues in the polyG tract (Fig. 2D). When the three pEX-encoded Pmp10 polypeptides were expressed as cro-β-galactosidase fusion proteins in *Escherichia coli*, it was evident that the pEX1-6 fusion protein differed in size relative to pEX1-1 and pEX1-2 (Fig. 3). This verified that a string of 15 guanines (pEX1-6) resulted in generation of a stop mutation. Sequence analysis showed that this stop was generated 12 bp downstream of the polyG tract. In strain TW183 a full-length Pmp10 was expressed since the gene contained 11 guanine residues and was in frame [15].

Finally, the presented results indicate that variation in the number of residues in the polyG tract of *pmp10* promotes the molecular mechanism for differential expression of *C. pneumoniae* Pmp10 during growth in cell culture. The benefit of modulating Pmp10 expression may be generation of functional diversity or antigenic variation. No other *C. pneumoniae* Pmp contains such a guanine-rich stretch, which is why regulation at the translational level of other Pmps has to take place differently.

Acknowledgements

This work was supported by the Danish Health Research Council (Grants 12-1620-1 and 9900750), the Danish Veterinary and Agricultural Research Council (Grant 20-3503-1) and the University of Aarhus Research Foundation. We thank Karin S. Sørensen and Inger Andersen for skillful technical assistance and Lisbet Wellejus Pedersen for linguistic assistance.

References

[1] Grayston, J.T. (1992) Infections caused by *Chlamydia pneumoniae* strain TWAR. Clin. Infect. Dis. 15, 757–763.
[2] Knudsen, K., Madsen, A.S., Mygind, P., Christiansen, G. and Birkelund, S. (1999) Identification of two novel genes encoding 97- to 99-kilodalton outer membrane proteins of *Chlamydia pneumoniae*. Infect. Immun. 67, 375–383.
[3] Vandahl, B.B., Birkelund, S., Demol, H., Hoorelbeke, B., Christiansen, G., Vandekerckhove, J. and Gevaert, K. (2001) Proteome analysis of the *Chlamydia pneumoniae* elementary body. Electrophoresis 22, 1204–1223.
[4] Grimwood, J., Olinger, L. and Stephens, R.S. (2001) Expression of *Chlamydia pneumoniae* polymorphic membrane protein family genes. Infect. Immun. 69, 2383–2389.
[5] Longbottom, D., Russell, M., Jones, G.E., Lainson, F.A. and Herring, A.J. (1996) Identification of a multigene family coding for the 90 kDa proteins of the ovine abortion subtype of *Chlamydia psittaci*. FEMS Microbiol. Lett. 142, 277–281.
[6] Tanzer, R.J., Longbottom, D. and Hatch, T.P. (2001) Identification of polymorphic outer membrane proteins of *Chlamydia psittaci* 6BC. Infect. Immun. 69, 2428–2434.
[7] Mygind, P.H., Christiansen, G., Roepstorff, P. and Birkelund, S. (2000) Membrane proteins PmpG and PmpH are major constituents of *Chlamydia trachomatis* L2 outer membrane complex. FEMS Microbiol. Lett. 186, 163–169.
[8] Tanzer, R.J. and Hatch, T.P. (2001) Characterization of outer membrane proteins in *Chlamydia trachomatis* LGV serovar L2. J. Bacteriol. 183, 2686–2690.
[9] Stephens, R.S., Kalman, S., Lammel, C., Fan, J., Marathe, R., Aravind, L., Mitchell, W., Olinger, L., Tatusov, R.L., Zhao, Q., Koonin, E.V. and Davis, R.W. (1998) Genome sequence of an obligate intracellular pathogen of humans: *Chlamydia trachomatis*. Science 282, 754–759.
[10] Kalman, S., Mitchell, W., Marathe, R., Lammel, C., Fan, J., Hyman, R.W., Olinger, L., Grimwood, J., Davis, R.W. and Stephens, R.S.

(1999) Comparative genomes of *Chlamydia pneumoniae* and *C. trachomatis*. Nature Genet. 21, 385–389.

[11] Read, T.D., Brunham, R.C., Shen, C., Gill, S.R., Heidelberg, J.F., White, O., Hickey, E.K., Peterson, J., Utterback, T., Berry, K., Bass, S., Linher, K., Weidman, J., Khouri, H., Craven, B., Bowman, C., Dodson, R., Gwinn, M., Nelson, W., DeBoy, R., Kolonay, J., McClarty, G., Salzberg, S.L., Eisen, J. and Fraser, C.M. (2000) Genome sequences of *Chlamydia trachomatis* MoPn and *Chlamydia pneumoniae* AR39. Nucleic Acids Res. 28, 1397–1406.

[12] Shirai, M., Hirakawa, H., Ouchi, K., Tabuchi, M., Kishi, F., Kimoto, M., Takeuchi, H., Nishida, J., Shibata, K., Fujinaga, R., Yoneda, H., Matsushima, H., Tanaka, C., Furukawa, S., Miura, K., Nakazawa, A., Ishii, K., Shiba, T., Hattori, M., Kuhara, S. and Nakazawa, T. (2000) Comparison of outer membrane protein genes *omp* and *pmp* in the whole genome sequences of *Chlamydia pneumoniae* isolates from Japan and the United States. J. Infect. Dis. 181 (Suppl. 3), S524–S527.

[13] Grimwood, J. and Stephens, R.S. (1999) Computational analysis of the polymorphic membrane protein superfamily of *Chlamydia trachomatis* and *Chlamydia pneumoniae*. Microb. Comp. Genomics 4, 187–201.

[14] Christiansen, G., Pedersen, A.S., Hjernø, K., Vandahl, B. and Birkelund, S. (2000) Potential relevance of *Chlamydia pneumoniae* surface proteins to an effective vaccine. J. Infect. Dis. 181 (Suppl. 3), S528–S537.

[15] Stephens, R.S. and Lammel, C.J. (2001) *Chlamydia* outer membrane protein discovery using genomics. Curr. Opin. Microbiol. 4, 16–20.

[16] Rockey, D.D., Lenart, J. and Stephens, R.S. (2000) Genome sequencing and our understanding of Chlamydiae. Infect. Immun. 68, 5473–5479.

[17] Christiansen, G., Østergaard, L. and Birkelund, S. (1994) Analysis of the *Chlamydia pneumoniae* surface. In: Chlamydial Infections: Proceedings of the Eighth International Symposium on Human Chlamydial Infection, pp. 173–176.

[18] Christiansen, G., Østergaard, L. and Birkelund, S. (1997) Molecular biology of the *Chlamydia pneumoniae* surface. Scand. J. Infect. Dis. 104 (Suppl.), 5–10.

[19] Puolakkainen, M., Parker, J., Kuo, C.C., Grayston, J.T. and Campbell, L.A. (1995) Further characterisation of *Chlamydia pneumoniae* specific monoclonal antibodies. Microbiol. Immunol. 39, 551–554.

[20] Christiansen, G., Madsen, A.S., Knudsen, K., Mygind, P. and Birkelund, S. (1998) Stability of the outer membrane proteins of *Chlamydia pneumoniae*. In: Chlamydial Infections: Proceedings of the Ninth International Symposium on Human Chlamydial Infection. International *Chlamydia* Symposium (Stephens, R.S. et al., Eds.), pp. 271–274.

[21] Wolf, K., Fischer, E., Mead, D., Zhong, G., Peeling, R., Whitmire, B. and Caldwell, H.D. (2001) *Chlamydia pneumoniae* major outer membrane protein is a surface-exposed antigen that elicits antibodies primarily directed against conformation-dependent determinants. Infect. Immun. 69, 3082–3091.

[22] Birkelund, S., Knudsen, K., Madsen, A.S., Falk, E., Mygind, P. and Christiansen, G. (1998) Differential expression of *Chlamydia pneumoniae* Omp4 and Omp5 after infection of C57-black mice? In: Chlamydial Infections: Proceedings of the Ninth International Symposium on Human Chlamydial Infection. International *Chlamydia* Symposium (Stephens, R.S. et al., Eds.), pp. 275–278.

[23] Christiansen, G., Boesen, T., Hjernø, K., Daugaard, L., Mygind, P., Madsen, A.S., Knudsen, K., Falk, E. and Birkelund, S. (1999) Molecular biology of *Chlamydia pneumoniae* surface proteins and their role in immunopathogenicity. Am. Heart J. 138, S491–S495.

[24] Clausen, J.D., Christiansen, G., Holst, U. and Birkelund, S. (1997) *Chlamydia trachomatis* utilizes the host cell microtubule network during early events of infection. Mol. Microbiol. 25, 441–449.

[25] Birkelund, S., Lundemose, A.G. and Christiansen, G. (1988) Chemical cross-linking of *Chlamydia trachomatis*. Infect. Immun. 56, 654–659.

[26] Birkelund, S., Lundemose, A.G. and Christiansen, G. (1989) Characterization of native and recombinant 75-kilodalton immunogens from *Chlamydia trachomatis* serovar L2. Infect. Immun. 57, 2683–2690.

[27] Longbottom, D., Russell, M., Dunbar, S.M., Jones, G.E. and Herring, A.J. (1998) Molecular cloning and characterization of the genes coding for the highly immunogenic cluster of 90-kilodalton envelope proteins from the *Chlamydia psittaci* subtype that causes abortion in sheep. Infect. Immun. 66, 1317–1324.

[28] Longbottom, D., Findlay, J., Vretou, E. and Dunbar, S.M. (1998) Immunoelectron microscopic localisation of the OMP90 family on the outer membrane surface of *Chlamydia psittaci*. FEMS Microbiol. Lett. 164, 111–117.